



## A PARTICULATE COMPLEX FOR ADMINISTERING NUCLEIC ACID INTO A CELL

### BACKGROUND OF INVENTION

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This invention concerns particulate complexes and their use for administering a nucleic acid molecule into a cell.

10 Many systems for administering active substances into cells are already known, such as liposomes, nanoparticles, polymer particles, immuno- and ligand-complexes and cyclodextrins (Drug Transport in antimicrobial and anticancer chemotherapy. G. Papadakou Ed., CRC Press, 1995). However, none of these systems has proved to be truly satisfactory for the in vivo transport of nucleic acids such as, for example, deoxyribonucleic acid (DNA).

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Satisfactory in vivo transport of nucleic acids into cells is necessary for example, in gene therapy. Gene therapy is the transfection of a nucleic acid-based product, such as a gene, into the cells of an organism. The gene is expressed in the cells after it has been introduced into the organism.

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Several methods of cell transfection exist at present. These methods can be grouped as follows:

- use of calcium phosphate, microinjection, protoplasmic fusion;
- electroporation and injection of free DNA.
- 25 - viral infection;
- synthetic vectors.

Methods in the first group are not applicable to in vivo transfection. As a result, most initial clinical trials of gene therapy taking place today are based upon the utilization of retroviral or adenoviral vectors. Examples of viral vectors that have been tried include retroviral, herpes virus, and adenoviral vectors. These retroviral vectors can be effective in stably transfecting heterologous genes into some cells for expression. However, clinical utilization of vectors of viral origin appears

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problematic because of their specificity, immunogenicity, high production costs, and potential toxicity.

Electroporation and injection of free DNA offer a useful alternative. These  
5 methods are, however, relatively ineffective, and limited to local administration only.

There is increasing interest in the use of synthetic vectors, such as lipid or polypeptide vectors. Synthetic vectors appear to be less toxic than the viral vectors.

10 Among synthetic vectors, lipid vectors, such as liposomes, appear to have the advantage over polypeptide vectors of being potentially less immunogenic and, for the time being, more efficient. However, the use of conventional liposomes for DNA delivery is very limited because of the low encapsulation rate and their inability to compact large molecules, such as nucleic acids.

15 The formation of DNA complexes with cationic lipids has been studied by various laboratories (Felgner et al., PNAS 84, 7413-7417 (1987); Gao et al., Biochem. Biophys. Res. Commun. 179, 280-285, (1991); Behr, Bioconj. Chem. 5, 382-389 (1994)). These DNA-cationic lipid complexes have also been designated in the past  
20 using the term lipoplexes (P.Felgner et al., Hum. Genet. Ther., 8, 511-512, 1997). Cationic lipids enable the formation of relatively stable electrostatic complexes with DNA, which is a polyanionic substance.

The use of cationic lipids has been shown to be effective in the transport of  
25 DNA in cell culture. However, the in vivo application of these complexes for gene transfer, particularly after systemic administration, is poorly documented (Zhu et al., Science 261, 209-211 (1993); Thierry et al., PNAS 92, 9742-9746 (1995); Hofland et al., PNAS 93, 7305-7309 (1996)).

30 Cationized polymers have also been investigated as vector complexes for transfecting DNA. For example, vectors called "Neutraplexes" containing a cationic polysaccharide or oligosaccharide matrix have been described in U.S. Application

Serial No. 09/126,402 owned by Biovector Therapeutics of Toulouse, France. Such vectors also contain an amphiphilic compound, such as a lipid.

Chitosan conjugates having pendant galactose residues have also been investigated as a gene delivery vector. See Murata et al., "Possibility of Application of Quaternary Chitosan Having Pendant Galactose Residues as Gene Delivery Tool," *Carbohydrate Polymers*, 29(1):69-74 (1996); Murata et al., "Design of Quaternary Chitosan Conjugate Having Antennary Galactose Residues as a Gene Delivery Tool," *Carbohydrate Polymers* 32:105-109 (1997). Chitosan is cationic natural polysaccharide. However, chitosan is strongly charged. Therefore, chitosan will complex too strongly to the nucleic acid to permit the proper release of the nucleic acid when reaching the target cells.

Galactosylated polyethyleneimine/DNA complexes have also been investigated. See Bettinger, et. al., "Size Reduction of Galactosylated PEI/DNA Complexes Improves Lectin-Mediated Gene Transfer into Hepatocytes," *Bioconjugate Chem.*, 10:558-561 (1999). However, such complexes rely upon a decrease in pH in lysosomes in order to release the DNA. Therefore, the mechanism cannot be extended to in vivo applications.

Therefore, there is a need for an improved particulate vector for administering a nucleic acid molecule into a cell.

### **SUMMARY OF INVENTION**

These and other inventions, as will be apparent to those having ordinary skill in the art, have been achieved by providing a particulate complex comprising a nucleic acid and a biodegradable cationized polyhydroxylated molecule, wherein the molecule has a charge up to approximately 1.0 meq/g.

## **BRIEF DESCRIPTION OF DRAWINGS**

Figure 1 is a graph demonstrating  $\beta$ -galactosidase expression in muscle with and without use of the complexes of the invention.

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## **DETAILED DESCRIPTION OF THE INVENTION**

The inventors have surprisingly discovered that a particulate complex between a nucleic acid molecule and a biodegradable cationized polyhydroxylated molecule provide advantages for transfecting a nucleic acid molecule into a cell. The charge on the vector should be sufficient to stably bind the nucleic acid. At the same time, the charge should remain low enough to allow for the necessary release of the nucleic acid molecule after reaching the interior of the cell.

“Nucleic acid” is defined as any single or double-stranded polynucleotide. Nucleic acid includes, for example, double or single stranded DNA, RNA or a mixture thereof. The nucleic acid can include natural or chemically modified sequences, or derivatives thereof. The nucleic acid can also be a mixture of different nucleic acids.

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The polynucleotide can be any size, depending on its purpose. The term "polynucleotide" as used herein, includes RNA or DNA sequences of more than one nucleotide in either single chain, duplex or multiple chain form. The polynucleotide may, for example, be an oligonucleotide. An oligonucleotide is a short length of single stranded polynucleotide chain, usually less than 30 residues long. The polynucleotide can also be much longer, with no upper limit.

The polynucleotide preferably includes the structural (coding) region of a gene. The polynucleotide may also encode signal sequences, such as promoter regions, operator regions, translocation signals, termination regions, combinations thereof or any other genetically relevant material. The gene being transfected can include only the structural region, and rely upon the non-structural regions existing in

the DNA of the cell being transfected. The polynucleotide can also encode only a signal sequence, if desirable. Examples of oligonucleotides which can be transfected are antisense oligonucleotides (DNA and RNA), ribozymes, and triplex-forming oligonucleotides. Optionally, the nucleic acid can be naked or can be part of a vector,  
5 other than the particulate complex of the invention.

The nucleic acid is complexed to a cationized polyhydroxylated molecule. Preferred polyhydroxylated molecules include, for example, saccharides, polyglycols, polyvinyl alcohol, polynoxylin, or polyethylene glycol (PEG). Saccharides include  
10 monosaccharides, oligosaccharides, and polysaccharides. The saccharides can be natural or synthetic.

Examples of polysaccharides include, starch, glycogen, amylose, and amylopectin. Examples of oligosaccharides include maltose, maltodextrin, lactose,  
15 and sucrose. Examples of monosaccharides include, galactose, mannose, fucose, ribose, arabinose, xylose, and rhamnose.

Glucidex is an example of a maltodextrin that can be used in the complex of the invention. Glucidex is referred to by number, which corresponds to the size of the  
20 molecule. For example, as shown in Table I in Example 1, Glucidex 2 has an average molecular weight of 10 kDa; Glucidex 6, made up of sixteen sugar units, has an average molecular weight of 3 kDa; Glucidex 12 has an average molecular weight of 1.4 kDa, and Glucidex 21 has an average molecular weight of 0.8 kDa.

The polyhydroxylated molecule can be cationized by grafting thereto a suitable cationic moiety. Examples of such cationic moieties include secondary or tertiary amino groups, quaternary ammonium ions, or a combination thereof.  
25 Glycidyl trimethylammonium (GTMA) is a preferred cationic group.

The cationized polyhydroxylated molecule is biodegradable. "Biodegradable" means that the molecule is able to be degraded by a hydrolytic enzyme naturally present in mammals in order to obtain fragments which are metabolized and/or  
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eliminated from the body. Examples of such enzymes include glycosidases, amylase, and glucosaminidase.

5 The cationized polyhydroxylated molecule has a positive charge up to approximately 1.0 meq/g. The charge may be as low as 0.001 meq/g, preferably 0.01 meq/g, and more preferably 0.1 meq/g. Molecules which have a charge greater than 1.0 meq/g will not be acted upon by hydrolytic enzymes and therefore are not biodegradable. Therefore, polyhydroxylated molecule does not include chitosan, quaternarized chitosan, or DEAE-Dextran.

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The optimal charge on the polyhydroxylated molecule will vary according to the size of the molecule and the nature of the nucleic acid to be grafted. The optimal charge can be determined by one of ordinary skill in the art. It is preferred that the polyhydroxylated molecule has a charge between approximately 0.1 and  
15 approximately 0.85 meq/g. In the case of GTMA and Glucidex, such charge expressed in meq/g corresponds to 1 to 10 moles of GTMA grafted per mole of Glucidex 2, or 0.3 to 3 moles of GTMA grafted per mole of Glucidex 6.

It is preferred that the cationized polyhydroxylated molecule have a molecular  
20 weight of between about 0.18 KDa and 1,000 KDa, more preferably between approximately 0.5 KDa and approximately 500 KDa.

The cationized polyhydroxylated molecule and nucleic acid are combined to form the particulate complex of the invention. The polyhydroxylated molecule and  
25 nucleic acid can be combined or grafted by methods known in the art. Because of their opposite charges, the polyhydroxylated molecule and the nucleic acid can be combined, for example, by simply mixing them in a solution. The order of mixing is not critical. For instance, saccharide powder can be solubilized in a saccharide solution. Additional steps can be used in the process, e.g. homogenization,  
30 lyophilization, concentration, evaporation, and ultrafiltration.

The particulate complex optionally includes a lipid component. In a preferred embodiment, the particulate complex lacks a cationic lipid component.

The particulate complex can include nucleic acids and biodegradable cationized polyhydroxylated molecules of various sizes. Therefore, the molecular weight of the particulate complexes of the invention will vary. The preferred size of the particulate complex as a whole is between approximately 100 nm to approximately 10  $\mu$ m, more preferably between 200 nm and 1  $\mu$ m.

The global charge of the particulate complexes of the invention is the result of the relative number of positive to negative charges, and can be described in terms of charge ratio. In this specification, a charge ratio is defined in accordance with Felgner, et al. "Nomenclature for Synthetic Gene Delivery Systems," *Human Gene Therapy*, 8:511-512(1997):

$$\text{Charge ratio} = \frac{\text{Positive charge of polyhydroxylated molecule in meq/g} \times \text{Mass (g)}}{\text{Negative charge of nucleic acid in meq/g} \times \text{Mass (g)}}$$

The positive charge of the polyhydroxylated polymer includes any cationic constituents. The negative charge of the nucleic acid includes any other anionic constituents. The charge ratio can also be expressed in terms of a percentage by multiplying the resulting fraction by 100. The charge ratio is expressed in this manner in Figure 1.

The zeta potential of a solution comprising the particulate complexes is an experimental parameter that is directly correlated to the cationized polyhydroxylated molecule/nucleic acid charge ratio. When the charge ratio is <1, the zeta potential is negative, which indicates a negatively charged surface on the particles. Alternatively, when this charge ratio is >1, the zeta potential is positive, which indicates a positively charged surface on the particles. Experimentally, the zeta potential, expressed as mV,

is indicative of the particle charge ratio. The zeta potential can be determined by a zeta potential analyzer.

5 The particulate complexes of the present invention may be positive or negative. The choice of a positive or negative complex is guided by the route of administration. In case of intravenous administration, a negative complex is more appropriate. For mucosal administration, a positive complex is preferred.

10 In a preferred embodiment, the complex has a charge ratio of cationized polyhydroxylated molecule to nucleic acid between approximately 0.3 to 1, wherein the complex is globally negative. In another preferred embodiment, the complex has a charge ratio of cationized polyhydroxylated molecule to nucleic acid between 1 to approximately 20, wherein the complex is globally positive.

15 It has been discovered that there is a close relationship between the charge ratio of the particulate complex, and the kinetics of release of the nucleic acid. The kinetics of the release of the nucleic acid, in turn, affects the efficacy of transfection of the released nucleic acid. The optimal charge ratio for each complex can be determined by a person of ordinary skill in the art, within the parameters set forth  
20 above.

When the complex is globally positive, there is more cationized polyhydroxylated molecule than is necessary to fully complex with the nucleic acid. This excess amount of cationized polyhydroxylated molecules is free and not grafted  
25 to the nucleic acid.

In a separate preferred embodiment, a solution is provided that includes a globally positive complex as described above and further includes excess polyhydroxylated molecule not grafted to nucleic acid. Without being bound by  
30 theory, it is believed that the excess polyhydroxylated molecule, such as a polysaccharide, may act as an enhancer for transfection. This may be due to the



interaction of the polyhydroxylated molecule or its degradation products with DNA and with the cellular membranes, enhancing the penetration of DNA into the cells.

5 A method is also provided for protecting a nucleic acid molecule when transfecting the nucleic molecule into a cell. The method includes complexing the nucleic acid with a cationized polyhydroxylated molecule to form a particulate complex as described above. "Protecting" means, for example, that the nucleic acid is prevented from being degraded or substantially degraded by, for example, a nuclease before the nucleic acid enters the cell. Without being bound by theory, it is believed  
10 that the cationized polyhydroxylated molecule protects the nucleic acid from enzymes or other sources of degradation.

15 In another separate embodiment, a method is provided for administering a nucleic acid molecule into a cell. The administration into the cell can occur ex vivo or to a mammalian cell in vivo. The method includes complexing the nucleic acid with a cationized polyhydroxylated molecule to form a particulate complex as described above. The particulate complex is then utilized in transfecting the nucleic acid molecule into a cell by known means.

20 In one embodiment, the nucleic acid molecule encodes a peptide or protein that shares at least one epitope with an immunogenic protein found on a pathogen. The pathogen may be, for example, a virus, bacteria, or protozoa. Examples of viral pathogens include human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus, HAV; hepatitis B virus, HBV;  
25 hepatitis C virus, HCV; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, CMV; Epstein-Barr virus, EBV; rhinovirus; and, coronavirus. Examples of bacteria include meningococcus, tuberculosis, streptococcus, and tetanus. Examples of protozoa include malaria or Trypanosoma. The complex is administered to the mammal so as to induce an  
30 immune response. The method is used for non-pathogen mediated mammalian pathologies where modulation of the immune response is important. Some examples

of non-pathogen mediated pathologies include cancer, autoimmune disease, and allergies.

5 The particulate complex may be administered to the mammal by any known means. For example, methods of administration can include mucosal, intratumoral, pulmonary, intravenous, intramuscular, intraparietal, intraocular, cutaneous, intradermal, subcutaneous, or a combination thereof.

10 The mammal treated in accordance with the method of the invention may be any mammal, such as farm animals, pet animals, laboratory animals, and primates, including humans. Farm animals include, for example, cows, goats, sheep, pigs, and horses. Pet animals include, for example, dogs and cats. Laboratory animals include, for example, rabbits, mice, and rats.

15 In another embodiment, the nucleic acid comprises at least the coding region of a therapeutic protein in order to synthesize the therapeutic protein in the cell. Some examples of therapeutic proteins include enzymes, hormones, antigens, clotting factors, regulatory proteins, transcription factors and receptors. Some specific examples of therapeutic proteins include erythropoietin, somatostatin, tissue  
20 plasminogen activator, factor VIII, etc. The nucleic acids could be designed to obtain an intracellular oligonucleotide, such as ribozymes, antisense, and gene transcripts. In this embodiment, the nucleic acid comprises at least the coding region of an oligonucleotide used to inhibit expression of a gene.

25 In a separate embodiment, the particulate complex is administered in a pharmaceutical composition. The pharmaceutical composition may be manufactured by known means and can include typical ingredients. For example, the pharmaceutical composition can include a pharmaceutically acceptable diluent or carrier, a buffer, a preserving or stabilising agent, an adjuvant, and/or an excipient.

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In a preferred embodiment, the pharmaceutical composition further includes a transfection enhancer. Examples of transfection enhancers include lipids, detergents, enzymes, peptides, or enzyme inhibitors.

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## EXAMPLES

### **Example 1: Preparation of biodegradable cationized saccharides having a charge between 0.2 and 1 mEq/g**

10 Twenty grams of maltodextrins of various molecular weight (Glucidex 2, Glucidex 6, Glucidex 12, Glucidex 21, Roquette, Lille, France) or amylopectin (Waxilys 200, Roquette) were dispersed in 2N NaOH as indicated in Table I. When the suspension was homogeneous, glycidyl trimethylammonium (GTMA) chloride (Fluka, Saint Quentin Fallavier, France) was added. The degree of ionic grafting on  
15 the saccharide was adjusted by varying amount of glycidyl trimethyl ammonium chloride (Table I). This reaction lead to grafting of 3-(N, N, N trimethylamino)-2-ol-1-propyloxy groups on the sugars.

The reaction mixture was stirred for 5 hours at room temperature. The  
20 solution of grafted saccharides was then brought to pH between 5 and 7 with concentrated acetic acid and then dispersed by addition of distilled water.

To remove all the salts and reaction by-products, the suspension was ultrafiltered (tangential ultrafiltration on Minisette system, Filtron, Pall Gelman  
25 Sciences) with a membrane having an appropriate cutoff according to the molecular weight of the polymer (see Table I). Smaller molecular weight (Glucidex 12 and Glucidex 21) polymers were precipitated by absolute ethanol.

The suspension polymers were sterilized by filtration through 0.2  $\mu$ m  
30 polyethersulfone membrane (SpiralCap® capsule, Pall Gelman Sciences). The grafting yield was determined by nitrogen elemental analysis by proton NMR. The results are presented in table I.

Table I

Saccharide	2N NaOH (ml)	Average molecular weight (daltons)	GTMA chloride (g)	Molecular cutoff (daltons)	Charge (mEq/g)
Glucidex 2	40	10,000	1.44	30.000	0.30
	40	10,000	2.34	30.000	0.46
Glucidex 6	40	3,000	1.44	3.000	0.30
	40	3,000	2.34	3.000	0.45
	40	3,000	4.68	3.000	0.85
Glucidex 12	30	1,400	1.70	---	0.32
	30	1,400	3.74	---	0.60
Glucidex 21	20	800	4.68	---	0.76
Waxilys 200	60	800,000	3.12	100.000	0.54

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### Example 2: Preparation of DNA / biodegradable cationized saccharide complexes

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DNA / biodegradable cationized saccharide complexes were formed by mixing a solution containing 100 µg DNA with the required quantity of cationized saccharides in a final volume of 1 ml under vortex stirring. The quantity of added cationized saccharides was dependent on the required DNA/polymer ratio. After 30 min. incubation at room temperature, 1 ml of complex solution was mixed with 125 µl acetate buffer 200 mM pH 5.3. The resulting mixture was homogenized with a vortex mixer and stored at 4°C.

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#### *Characteristics of the DNA / biodegradable cationized saccharides complexes.*

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The visual appearance of the complexes was clear and homogeneous. Their characteristics are summarized in Table II. DNA / biodegradable cationized

saccharide complexes appeared to range from 60 to 3,000 nm in diameter as determined by light scattering measurement (Coulter N4 SD).

Table II

DNA	Polymers	Charge mEq/g	Charge ratio Polymers/DNA	Size	Zeta potential mV
100µg	Glucidex 6	0.45	2	74 nm	+ 25
100µg	Glucidex 6	0.45	4	70 nm	+ 40
100µg	Glucidex 6	0.85	4	90 nm	+ 30
100µg	Glucidex 2	0.46	0.5	180 nm	- 20
100µg	Glucidex 2	0.46	2	120 nm	+ 22
100µg	Glucidex 2	0.46	4	115 nm	+ 28
100µg	Glucidex 2	0.50	20	70 nm	+ 26
100µg	Glucidex 2	0.3	4	120 nm	+ 18
100µg	Waxilys	0.54	0.5	0.5-2 µm	ND <sup>a</sup>
100µg	Waxilys	0.54	2	1- 2 µm	ND

5 <sup>a</sup>ND : not determined

10 The percentage of DNA association was estimated by 1% agarose gel, TAE 1X. 20µl of the formulation were mixed with 2µl of loading solution 10X (glycerol 50%, bromophenol blue 0.025%), then 20µl of the resulting solution were loaded per well. The calculated quantity of DNA loaded was 1.6 µg/well. As a control, the same quantity of DNA has been loaded. After 40 min migration of the gel at 90 V, the gel was stained in a BET bath before visualization under U.V. light.

15 No migration of DNA was detected for the loaded DNA/ cationized saccharide complexes tested. Migration was only observed for the free DNA not grafted to a cationized saccharide. These results demonstrate that 100% of the initial DNA input dose was complexed by the saccharide.

### 20 **Example 3: Biodegradability of the cationized saccharides, and liberation of the entrapped DNA**

The biodegradability of the DNA/ cationized saccharides complexes was assayed by an in vitro degradation assay. 200µl of formulations were added to 40 µl

of amylase cocktail (1 mg/ml  $\alpha$ -amylase, 1 mg/ml amyloglucosidase in citrate buffer 100mM pH5). After overnight incubation under rotative agitation at room temperature , 20  $\mu$ l of the treated samples were loaded on 1% agarose gel.

When the amylase was omitted, no migration of DNA was detected for the loaded DNA/ cationized saccharide complexes. When the amylase was added, a significant part of the DNA migrated inside the gel. For the complexes having a low saccharide/DNA ratio, all the DNA was recovered and migrated at the same position as free DNA.

These results demonstrate that the polymer is biodegradable, which permits DNA release. Moreover, after release, no modification of DNA could be detected. As an example, no change of supercoiled/relaxed ratio is detected, which indicates that no nicking of DNA occurs during the formation of the particles.

#### **Example 4: In vivo transfection studies**

##### *I Materials and Methods*

##### *Plasmid DNA*

Gene transfer studies were carried out with pCMV $\beta$  plasmid DNA (Clontech) coding for  $\beta$  galactosidase. The plasmid DNA was purified by double chloride cesium gradient centrifugation (BioServe Biotechnologies, Ltd, USA) and resuspended in purified water. The concentration of DNA was 4.7 mg/ml as calculated based on absorbance of ultraviolet light (OD 260). Endotoxine level was 2.5 IU/mg as determined by the Limulus assay (Charles River, France). DNA solutions were stored at  $-20^{\circ}\text{C}$  until required for use. DNA was administered either as pure plasmid DNA on saline (naked DNA) or formulated with the biodegradable cationized saccharides.

### *DNA/Biodegradable cationized saccharide complexes*

The biodegradable cationized saccharide was synthesized as described above in Example 1. The DNA/glu2 and DNA/glu6 complexes were prepared as described  
5 above in Example 2.

### *In vivo gene transfer*

#### *Animals.*

All experiments were carried out using 8-9 week-old female BALB/c mice  
10 (Janvier, France) with 4 mice per experimental or control group.

#### *Intramuscular administration.*

Each animal received one intramuscular injection of 8 µg of naked or formulated DNA in a total volume of 100 µl in each quadriceps. The injections was  
15 made using a 27x1/2 gauge needle fitted with a polyethylene tubing which limited the penetration to 2 mm.

#### *Evaluation of reporter gene expression.*

The entire quadriceps muscle was collected from each mouse leg at day 7  
20 postinjection. Muscles were frozen in liquid nitrogen immediately after collection and stored in 2.0 ml Eppendorf tubes at -80°C. Frozen muscles were individually pulverized into a fine powder by hand grinding with a dry ice-chilled porcelain mortar and pestle and the powder was stored in the same tube at -80°C until extraction. One ml of β-galactosidase lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton  
25 X-100, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride and 5 µg/ml leupeptin) was added. The latter three components were added just before use. The samples were vortexed for 15 min, frozen and thawed three times using alternating liquid nitrogen and 37°C water baths, and centrifuged for 5 min at 13.000 RPM. The supernatant was transferred to another 1.5 ml eppendorf tube and stored at -80°C until  
30 tested for β galactosidase enzyme assays.

β galactosidase enzyme assays using MUG (Sigma, France) as a β-galactosidase substrate were performed in a reaction buffer containing 25 mM Tris-HCl (pH 7.5); 125 mM NaCl; 1 mM DTT; and 2 mM MgCl<sub>2</sub>. Just before use, MUG substrate (prepared as a 20 mg/ml slurry in ethanol) was added to a final concentration of 100 μg/ml.

Standards were prepared by adding known quantities of purified β-galactosidase (Promega) in 50 μl of control muscles extract supernatant (over the range of 200 pg to 200 ng in 50μl). Samples were assayed by addition of 200 μl of complete reaction buffer to 50 μl of sample in a 1.5 ml eppendorf tube and incubated at 37°C for 1 hour. The reactions were stopped by adding 50 μl of cold 25% trichloroacetic acid, chilled on ice for 5 min and clarified by centrifugation for 2 min at room temperature. 200 μl aliquots of each sample were added to 2 ml of glycine/carbonate buffer, vortexed, and read in a spectrofluorimeter using 366 nm excitation and 442 nm emission.

Protein concentrations of muscle extracts were determined using the microBCA assay (Pierce). β galactosidase enzyme concentration present in the sample was measured and expressed as ng β galactosidase /mg of total protein after normalization with β galactosidase standard curve and protein concentrations.

## II *Results*

The results are shown in Fig. 1. DNA formulated with cationic Glucidex 2 and Glucidex 6 and administrated intramuscularly allows high levels of β galactosidase expression in muscle. The highest expression was obtained with DNA/glu2 at the charge ratio of 20 and DNA/glu6 at the charge ratio of 2. Also, an increased amount of expression was observed when the charge ratio was progressively increased for glu2. Most importantly, the amount of expression with DNA/glu6 at the charge ratio of 2 was higher than with naked DNA.



## Example 5: Immunological study

### I *Materials and Methods*

#### *Plasmid DNA*

- 5 Immunization studies were carried out with pCMV $\beta$  plasmid DNA (Clontech) coding for  $\beta$  galactosidase described in Example 4.

#### *DNA/Biodegradable cationized saccharide formulations.*

- 10 The biodegradable cationized saccharide was synthesized as described above in Example 1. The DNA/Glucidex 2-GTMA and DNA/Glucidex 6-GTMA formulations were prepared as described above in Example 2.

#### *DNA immunization*

##### *Animals.*

- 15 Immunization experiments were carried out using 8-9 week-old female BALB/c mice (Janvier, France) with 4 mice per experimental or control group

##### *Intramuscular administration.*

- 20 Each animal received 3 intramuscular injections at 3 week-intervals of 8  $\mu$ g of naked or formulated DNA in a total volume of 100  $\mu$ l (50  $\mu$ l in each quadriceps). The injections was made using a 27x1/2 gauge needle fitted with a polyethylene tubing which limited the penetration to 2 mm.

- 25 *Collection of blood samples.*

Peripheral blood was collected by retro-orbital puncture 2 weeks after each injection.

##### *Antibody-assays.*

- 30 Serological responses were measured by enzymes-linked immunosorbant assay (ELISA). Maxisorb microtiter wells (Nunc, Denmark) were coated with 50  $\mu$ l of recombinant  $\beta$  galactosidase protein (Roche Diagnostics, France) at 2  $\mu$ g/ml in

PBS for 1 night at 4°C. Wells were blocked with 5% dried milk in PBS for 1h and washed with 0.05% Tween-20 in PBS. Sera were diluted in PBS with 1% dried milk and 0.05% Tween-20. A 50 µl-sample of serum per well was incubated for 2h at 37°C before washing and addition of horseradish peroxidase-conjugated goat anti-  
5 mouse IgG (Sigma, France). After 1h-incubation and washing, 100 µl of phosphatase substrate system (BluePhos Microwell, Interchim, France) were added.

For determination of the levels of anti-β galactosidase IgG1 and IgG2a responses, peroxidase-conjugated anti-mouse IgG1 or IgG2a (Pharmingen),  
10 respectively, were substituted for the anti-mouse IgG described previously. The color developed was measured at 620 nm. Antibody titers were expressed as the reciprocal of the final dilution which gave an absorbance ≥ 0.2.

#### *Proliferation assay*

15 Splens of mice were collected 7 days after the third immunization. Single cell suspensions were prepared and cultured in flat bottom 96-well plates (Nunc) at a density of  $5 \times 10^5$  cells/well in complete RPMI Glutamax II medium (Gibco Brl) containing 10% (v/v) Foetal calf serum (Gibco Brl), 20 µM β-Mercaptoethanol, 10 mM Hepes buffer, penicillin, Streptomycin, sodium pyruvate. Purified β-  
20 galactosidase (Roche) was added to culture wells at varying concentration ( 0.1, 1, 10 µg/ml). Cells were cultured in medium alone or in presence of 5 µg/ml Concanavalin A as negative and positive controls, respectively.

After 3 or 5 days of in vitro restimulation with β-galactosidase antigene or  
25 conA, cell proliferation was measured by a standard BrdU ELISA colorimétric assay (Roche) according to manufacturer's instruction. Briefly, cells were incubated during 4 h with BrdU. The plates were then centrifuged at 1000 RPM, and the culture medium was gently removed in such a way that 50 µl of medium was let to avoid cell aspiration. 300µl of PBS were added per well to wash the cells. Plates were  
30 recentrifuged and 300µl of supernatant were removed. The remaining medium was evaporated and the cells dried using a rapidvap at 80°C during 1 hour. After DNA denaturation during 30 min with 100 µl /well of FixDenat solution , antibody anti –

BrDU-POD working solution was added and incubated during 90 min. After three washings, 100µl of substrate solution was added and incubated at room temperature until color development was sufficient for photometric detection (generally during 10 min). The reaction was stopped by adding 25 µl of 1M H<sub>2</sub>SO<sub>4</sub> solution to each well.

- 5 The absorbance of the samples was measured in an ELISA reader (Labsystem) at 450 nm.

#### *Cytokines measurements*

- 10 Spleens of mice were collected 7 days after the third immunization. Single cell suspensions were prepared and cultured in flat bottom 96-well plates (Nunc) at a density of  $5 \times 10^5$  cells/well in complete RPMI Glutamax II medium (Gibco Brl) containing 10% (v/v) Foetal calf serum (Gibco Brl), 20 µM β-Mercaptoethanol, 10 mM Hepes buffer, penicillin, Streptomycin, sodium pyruvate. Purified β-galactosidase (Roche) was added to culture wells at varying concentration (0.1, 1, 10
- 15 µg/ml). Cells were cultured in medium alone or in presence of 5 µg/ml Concanavalin A as negative and positive controls respectively. Supernatants were collected after 24 and 72 h of culture and store at -80°C until cytokines secretion could be analyzed. Production of IFN-γ and IL-4 from 24h and 72 h culture supernatant was measured by using ELISA kit Duo Set (R&D systems) according to manufacturer's instruction.

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#### *IFN-γ ELISPOT assay*

- Flat bottom multiscreen 96-well plate (Millipore) was coated overnight at 4°C with 75µl/well of a 5 µg/ml R4AG6 capture antibody (Pharmingen). After 3 washing steps with RPMI medium, the plate was saturated 2 hours at 37°C with 200µl/well of
- 25 complete RPMI medium with 10% FCS. After removal of the saturating medium, 100µl of cells suspension ( $5 \times 10^5$  cells) were added to the wells, and the plate was incubated during 24h at 37°C under humidified atmosphere with 5% CO<sub>2</sub>. After 5 washing steps with PBS-tween 0.05% buffer, 100µL/well of the 5µg/ml XMG1.2 biotinylated antibody diluted in PBS-tween 0.05% buffer were added. After 1 hour
- 30 incubation at 37°C, the plate was washed 5 times with PBS-tween 0.05%, and 100µl/well of extravidine-AP (SIGMA) diluted in PBS-tween 0.05% BSA 1% were added. After 1 hour incubation, the plate was washed 5 times and 100µl/well of AP-

substrate solution (Biorad) were added. After 30 min revelation, spot counting was done with binocular microscope.

#### *Cytotoxic T-cell assays*

5           The method used is based on the fluorimetric dosage of  $\beta$ -galactosidase release in the cell culture supernatant from stable  $\beta$ -galactosidase transfected target cells (Ohmori et al, 1992). The immune spleen cells were restimulated in flat bottom 24-well plates (Nunc) at a density of  $5 \times 10^6$  cells/well in complete RPMI Glutamax II medium (Gibco Brl) containing 10% (v/v) Foetal calf serum (Gibco Brl), 20  $\mu$ M  $\beta$ -  
10   Mercaptoethanol, 10 mM Hepes buffer, penicillin, Streptomycin, sodium pyruvate, and 20U:ml IL-2 (Roche) for 5 days in presence of 0.5  $\mu$ g/ml TPHPARIGL peptide. This synthetic monamer TPHPARIGL represents the naturally processed H-2L<sup>d</sup>-restricted epitope of  $\beta$ -galactosidase spanning amino acids 876-884 (Neosystem).

15           The target cells were BC- $\beta$ -gal cells which are mouse fibroblast stable transfected by - $\beta$ -galactosidase gene. The target cells were resuspended to  $10^4$  cells/well in flat bottom 96-well plates (Nunc) in 100  $\mu$ l of complete RPMI medium. The restimulated spleen cells were then added to the wells, in a ratio effector / target cells of 100/1, 30/1, 10/1, 3/1, 1/1. After 4 hour incubation, the culture medium was  
20   removed and mixed (vol./vol.) with 2X  $\beta$ -gal lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride and 5  $\mu$ g/ml leupeptin). 50 $\mu$ l was used for the dosage of  $\beta$ -galactosidase activity by fluorimetric assay as described above in Example 4.

#### 25   II.     Results

          Following the intramuscular administration of the DNA/cationized saccharides complexes, the mice developed antibodies against  $\beta$ -galactosidase protein in serum. Without being bound by theory, it is believed that a cellular immune response was induced by the DNA/cationized saccharide because the DNA/cationized saccharide  
30   complexes induce DNA transfection.